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Condensin Regulates the Stiffness of Vertebrate Centromeres

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When chromosomes are aligned and bioriented at metaphase, the elastic stretch of centromeric chromatin opposes pulling forces exerted on sister kinetochores by the mitotic spindle. Here we show that condensin ATPase activity is an important regulator of centromere stiffness and function. Condensin depletion decreases the stiffness of centromeric chromatin by 50% when pulling forces are applied to kinetochores. However, condensin is dispensable for the normal level of compaction (rest length) of centromeres, which probably depends on other factors that control higher-order chromatin folding. Kinetochores also do not require condensin for their structure or motility. Loss of stiffness caused by condensin-depletion produces abnormal uncoordinated sister kinetochore movements, leads to an increase in Mad2(+) kinetochores near the metaphase plate and delays anaphase onset.

INTRODUCTION

Centromeric chromatin is a special region of chromosomes that has important mechanical and signaling functions in mitosis (Pidoux and Allshire, 2005; Ekwall, 2007; Cheeseman and Desai, 2008; Vagnarelli *et al.*, 2008). In metaphase, pulling forces generated by interactions between spindle microtubules (MTs) and kinetochores are opposed by tension produced by centromeric chromatin stretch. Centromere and kinetochore tension and stretch are important for maintaining chromosome alignment (McIntosh *et al.*, 2002), stabilizing kinetochore microtubule (kMT) attachments (Nicklas and Koch, 1969), spindle checkpoint signaling (Musacchio and Salmon, 2007; McEwen and Dong, 2009), and also for the back-to-back orientation of sister kinetochores (Loncarek *et al.*, 2007). At least three independent factors have roles in the establishment of centromeric tension in metaphase: sister chromatid cohesion (Yeh *et al.*, 2008), the elastic properties of chromatin (Houchmandzadeh *et al.*, 1997; Almagro *et al.*, 2004; Marko, 2008), and the higher order structure of the centromeric chromatin.

Condensin is important for the architecture of mitotic chromosome arms (Coelho *et al.*, 2003; Hudson *et al.*, 2003; Hirota *et al.*, 2004; Hirano, 2006), but it also localizes to centromeres (Saitoh *et al.*, 1994; Gerlich *et al.*, 2006), where condensin I, but not condensin II was reported to have a role in stabilizing the structure (Gerlich *et al.*, 2006). It has recently been suggested that condensin could have a role in regulating the elastic behavior of centromeric chromatin. One study found that condensin I–depleted *Drosophila* chromosomes were unable to align at a metaphase plate, had distorted kinetochore structures, and lost elasticity of their centromeric chromatin (Oliveira *et al.*, 2005). However a similar study in human cells reported that although loss of condensin I caused kinetochores to undergo abnormal movements, these movements were bidirectional (e.g., reversible; Gerlich *et al.*, 2006).

Even after the publication of those results, the regulation and functional significance of centromere stretch remained unknown. An elegant study in budding yeast went on to find that chromatin structure sets the rest length of the centromere, but does not regulate its stretch (Bouck and Bloom, 2007). Here, we analyzed the movements of kinetochores, pericentromeres, and distal chromosome arms during metaphase in DT40 cells bearing a conditional knockout of SMC2, an essential subunit of condensin I and II. Our results reveal that condensin ATPase activity is required to regulate centromere stretch and that loss of condensin results in a mitotic delay accompanied by an increased number of Mad2-positive kinetochores on chromosomes aligned at the metaphase plate.

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MATERIALS AND METHODS

Cell Culture, Transfections, and Drug Treatments

The SMC2 conditional knockout cell line and SMC2:CENP-H:GFP_H2B:mRFP were cultured as previously described (Hudson *et al.*, 2003; Vagnarelli *et al.*, 2006). The SMC2 conditional knockout cell line containing a human X-derived minichromosome was described previously (Vagnarelli *et al.*, 2006).

The constructs GgMad2-green fluorescent protein (GFP) and GFP-hMCAK were obtained from T. Fukagawa (National Institute of Genetics, Tokyo) and from J. Swedlow (University of Dundee, United Kingdom), respectively.

Supplemental Material can be found at:
http://www.molbiolcell.org/content/suppl/2009/03/03/E08-11-1127.DC1.html

Stable transfectants were selected in 0.5 μg/ml puromycin or 25 μg/ml blasticidin for 10 d.

The construct GFP-GgCENPA was obtained by cloning GgCENPA into pEGFPC1 with a 17-amino acid linker.

The cell lines lacO:lacI-GFP in SMC2 conditional knockout background were described elsewhere (Vagnarelli *et al.*, 2006). The lacO:lacI-GFP CEN integration was used to prepare stable cell lines expressing the rescue construct SMC2-SBP wild-type or the mutant form of SMC2-SBP S1086R.

For Hec1 RNA interference (RNAi) a 21-mer oligonucleotide (ccagacugaggaagaaauudtdt) covering bases 1404-1424 downstream of the start codon of *Gallus gallus* Hec1 cDNA was used. A 21-mer oligonucleotide (cguacgcggaauacuucgadtdt) with no significant homology to any known chicken mRNA in the databases was used as control. The lacO:lacI-GFP cell line was grown in the presence of doxycycline for 8 h and then transfected with 10 μM of each small interfering RNA using the Nucleofector system (Amaxa, Cologne, Germany) and plated in complete medium plus doxycycline. The experiments were analyzed between 26 and 30 h after repression.

To calculate the interkinetochore distances in the absence of MTs, cells were treated for 2 h with colcemid at 0.5 μg/ml. The analysis of the lacO CEN movements in the presence of different drugs was conducted as follows. Cells were incubated for 2 h with 20 μM MG132 and then prepared for live cell imaging. For the nocodazole experiments cells were treated with 0.5 μg/ml nocodazole while imaging. ICRF-159 treatments were carried out for 3 h with 10 μg/ml the drug.

For MT drug experiments, cells were treated with 0.5 μg/ml nocodazole (Sigma, St. Louis, MO) or 40 nM paclitaxel (Taxol; Sigma) for the period of time indicated in the text.

Live Cell Imaging

Digital images were collected with a cooled CCD camera (Orca ER; Hamamatsu, Bridgewater, NJ) coupled to a Yokogawa spinning disk confocal unit (CSU10; Perkin Elmer, Norwalk, CT), which was attached to an inverted microscope (TE300; Nikon, Melville, NY) with a 100× 1.4 NA plan-Apochromatic differential interference contrast objective. Image acquisition and microscope shutter were controlled by Metamorph software (Universal Imaging, West Chester, PA) on a PC computer. Stage temperature was maintained at ~37°C using an air curtain incubator (ASI 400; Nevtek, Burnsville, VA). Fluorescence images were acquired at 488 nm at a single focal plane with an exposure time of 1500 ms every 4 s.

Image Analysis and Kymographs

The movement of paired lacO:lacI-GFP integrations was followed using the Track Objects tool in Metamorph after image calibration; whenever any of the spots was out of focus, the time point was removed from the raw movie. After exporting the coordinates (x,y) of the LacI spots to Excel (Microsoft, Redmond, WA), the angle between a line drawn through both spots, and the horizontal axis was calculated for each image in the stack. A custom-written program in MatLab (MathWorks, Natick, MA; created by J. Gatlin) was used to align and rotate the raw image stacks based on the calculated angle and the position of one of the two LacI spots. The aligned stack was then exported back to Metamorph where the Kymograph tool was used to build kymographs.

The distance of paired lacO:lacI-GFP integrations in live cell imaging movies was determined using the Measure Distance tool in Metamorph, and the values were exported to Excel. The distance values and the time were plotted in a line graph in Excel.

The measurements of the lacO integration in fixed samples were carried out on a microscope (Model IX-70; Olympus) controlled by DeltaVision Softworks (Delta Data Systems, Cornwells Heights, PA) using the SoftWorks tool "Measure Distance." The cells were selected according to the following criteria: 1) perfectly aligned metaphase plates and 2) both lacO integrations visible in the same focal plane. Fluorescence images were acquired at 488 nm on a single focal plane, and the measurements were determined.

The distance between the centroids of Hec1 and CENP-A was obtained by image calibration and line profile analyses (Metamorph) on projected three-dimensional (3D) stacks. After obtaining the graph, the distance between the highest peaks for Hec1 and GFP-CENP-A was calculated.

The spindle pole separation measurements were carried out on cells immunostained for γ-tubulin with DNA visualized using DAPI. Measurements were only considered in cells where the metaphase plate was clear and both γ-tubulin stained poles were in the same focal plane. These selected cells were imaged in a single focal plane at 568 nm, and the distance was measured.

Quantification of the CENP-H:GFP Molecules

The quantification was determined by quantitative fluorescence of CENP-H:GFP (in cells where GFP was knocked into the endogenous single copy CENP-H gene) relative to the copy number of 8 Ndc80 molecules per budding yeast kinetochore according to the method previously described (Joglekar *et al.*, 2006).

Indirect Immunofluorescence and Microscopy

Immunostaining in DT40 cells was conducted as previously described (Vagnarelli *et al.*, 2006). Antibody incubation was done in 1% BSA-phosphate-buffered saline (PBS) for 1 h at 37°C. The following antibodies were used: mouse anti-α-tubulin at 1:1000 (Sigma), mouse anti-γ-tubulin at 1:1000 (Sigma), anti-phospho H3T3 (Abcam, Cambridge, MA) at 1:200, anti-Hec1 (T. Fukagawa, National Institute of Genetics, Tokyo) at 1:200, anti-BubR1 at 1:500, anti-CENP-H at 1:200 (T. Fukagawa), anti-CENP-A at 1:2000 (T. Fukagawa), fluorescence-labeled secondary antibodies 1:200 (Jackson ImmunoResearch Laboratories, West Grove, PA).

TEEN experiments were performed as previously described (Hudson *et al.*, 2003 and see text). 3D data sets were collected with a DeltaVision system (Applied Precision, Issaquah, WA) and deconvolved using the standard algorithms in SoftWorX software. Images were either used as single planes or as quick projections.

Electron Microscopy

DT40 cells were attached to concanavalin A–coated grided coverslips. The cells were fixed with 2.5% electron microscopy (EM) grade glutaraldehyde in phosphate-buffered saline (PBS) and in some cases 1% Triton was added to the primary fixative to aid in visualizing kinetochore MTs. Metaphase cells were identified by fluorescence light microscopy and located on the finder grid. Cells were postfixed with osmium tetroxide, tannic acid, and uranyl acetate, dehydrated in a grade ethanol series and propylene oxide, and flat-embedded in Epon. Same-cell correlative light microscopy (LM)/EM methods were used to find metaphase cells after embedment. Serial sections 100 and 150 nm thick were cut, stained, and imaged on a Zeiss 910 transmission electron microscope (Thornwood, NY).

RT-PCR and Immunoblotting Analysis

To screen for $SMC2^{ON/OFF}$ lacO cell lines expressing the rescue construct SBP-$smc2^{WT}$ or the mutant form SBP-$smc2^{S1086R}$ cells were grown in absence ($SMC2^{ON}$) or the presence ($SMC2^{OFF}$) of doxycycline and harvested for RNA extraction. Total RNAs were reverse-transcribed using oligo-dT primers and Superscript reverse transcriptase (Invitrogen-BRL, Carlsbad, CA) under conditions recommended by the manufacturer. Amplification was carried out with the Taq DNA Polymerase (Roche, Indianapolis, IN) using either a specific forward primer located in the 5′UTR of the Gg SMC2 sequence (5′-ttcactgagggctcccttcg-3′) or a specific primer for the *SMC2-tetO* transgene (5′-aatggcattgaataacgg-3′). The reverse primer for both was specific for the Gg SMC2 sequence (5′-aatggcattgaataacgg-3′). RT-PCR products were then resolved in a 0.8% agarose gel, and expression of the transgene was detected by the appearance of fragments of 190 base pairs, whereas the SMC2-tetO driven cDNA generated a 120-base pairs fragment.

Immunoblotting was conducted as previously described (Ruchaud *et al.*, 2002) using anti-SMC2 (1:500), anti-SBP (1:300), or anti-β tubulin (1:10,000), followed by horseradish peroxidase–conjugated secondary antibodies (1:10,000, Amersham Pharmacia Biotech, Piscataway, NY), and results were visualized using the enhanced chemiluminescence protocol (Amersham).

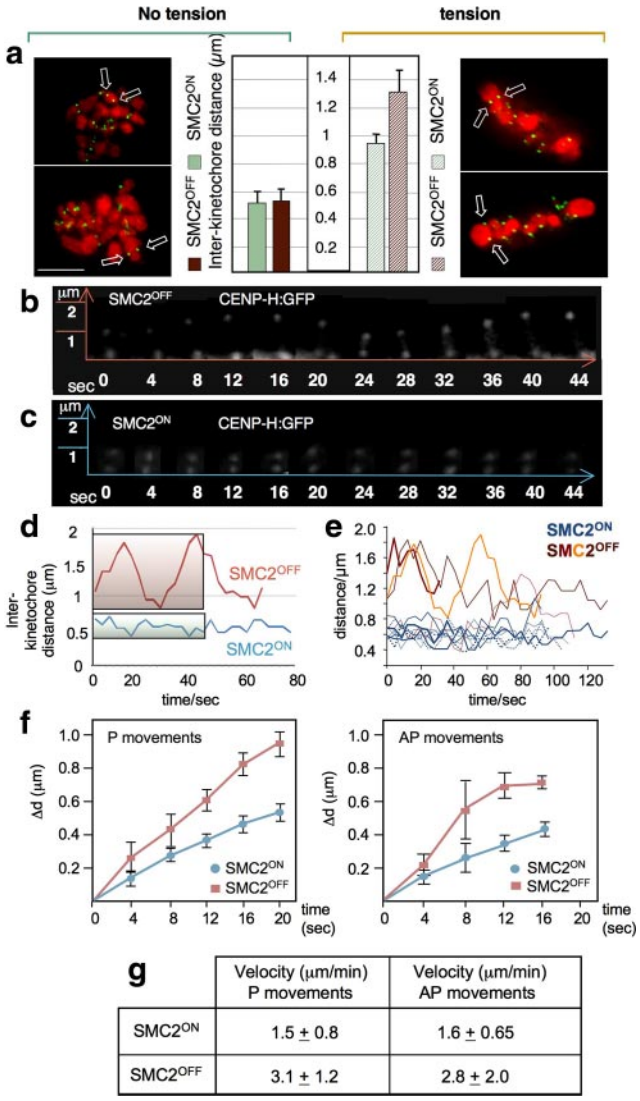
Chromosome Mis-segregation Analysis

An $SMC2^{ON/OFF}$ cell line containing a 2.7-Mb X-derived minichromosome was analyzed at 30 h after the addition (or not) of doxycycline. Cytochalasin D (Sigma) at 6 μg/ml was added 5 h before fixation in order to maintain the products of each segregation event within a common cytoplasm. Fixed cells were subjected to FISH with an X-specific α-satellite probe as previously described (Vagnarelli *et al.*, 2006).

RESULTS

We previously described a chicken DT40 B lymphocyte cell line in which the single copy SMC2 gene is disrupted, and cells are kept alive by an SMC2 cDNA expressed under control of a tetracycline-repressible promoter (Hudson *et al.*, 2003). In cells grown with doxycycline for 30 h ($SMC2^{OFF}$), no SMC2 protein or mRNA was detected by immunoblotting or RT-PCR, respectively (Hudson *et al.*, 2003; Vagnarelli *et al.*, 2006; see Supplementary Figure S1, a and b).

In $SMC2^{OFF}$ cells, mitotic chromosome condensation still occurs; however, the chromosome arms only reach 60% the normal level of compaction (Vagnarelli *et al.*, 2006). In those cells, the average distance between sister kinetochores at metaphase in fixed preparations was 1.4 times greater than that in $SMC2^{ON}$ cells (Figure 1a, right). However, after MT de-polymerization with colcemid, interkinetochore distances were indistinguishable between $SMC2^{ON}$ and



g	Velocity (μm/min) P movements	Velocity (μm/min) AP movements
$SMC2^{ON}$	1.5 ± 0.8	1.6 ± 0.65
$SMC2^{OFF}$	3.1 ± 1.2	2.8 ± 2.0

Figure 1. Condensin affects centromere stiffness but not rest length. (a) Interkinetochore distance of $SMC2^{ON}$ and $SMC2^{OFF}$ metaphase cells in the absence (+ colcemid) and presence of MTs. Arrows indicate sister kinetochore pairs. For the graph, n = 100. Green, CENP-H:GFP; red, H2BmRFP. Scale bar, 5 μm. (b and c) Stills from movies of a CENP-H:GFP cell line showing kinetochore movements (time lapse, 4 s): (b) $SMC2^{OFF}$; (c) $SMC2^{ON}$. (d) Quantification of kinetochore movements in $SMC2^{ON}$ (blue) and $SMC2^{OFF}$ (red) cells. The shaded areas show the regions of the curves represented in b and c. (e) Examples of typical kinetochore movements in $SMC2^{ON}$ (blue lines) and $SMC2^{OFF}$ (red lines) during metaphase. Time lapse, 4 s. (f) Velocities of poleward (P) and away from the pole (AP) kinetochore movements in $SMC2^{ON}$ (blue lines) and $SMC2^{OFF}$(red lines) cells during metaphase. (g) Average velocities of poleward (P) and away from the pole (AP) kinetochore movements in $SMC2^{ON}$ and $SMC2^{OFF}$cells during metaphase. For $SMC2^{ON}$ kinetochores, 30 P and 32 AP movements were analyzed; for $SMC2^{OFF}$ kinetochores, 12 P and 10 AP movements were analyzed.

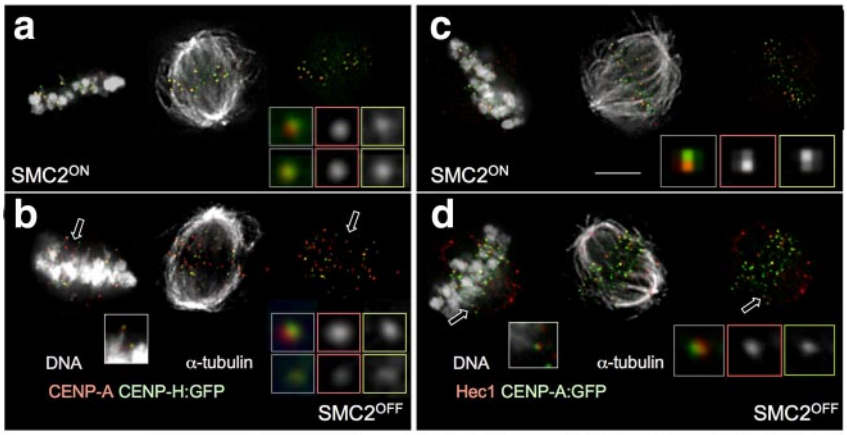
$SMC2^{OFF}$ cells (Figure 1a, left). Thus, condensin is not required for compaction of the heterochromatin between sister kinetochores, i.e., to set the "rest length" of the centromeric chromatin. However, those normally compacted centromeres lacking condensin respond abnormally to MT pulling forces. These findings confirm and extend a previous study in HeLa cells in which a MT-dependent abnormal mobility of kinetochores in metaphase was observed after condensin I RNAi (Gerlich *et al.*, 2006).

Condensin Depletion Affects the Dynamic Behavior But Not the Structure of Kinetochores in Mitosis

The core kinetochore protein CENP-H:GFP expressed from its own endogenous promoter was used to follow kinetochore movements in living cells. CENP-H is encoded by a single copy essential gene present on the Z sex chromosome. In vertebrate mitotic cells, the oscillations of sister kinetochores of bioriented chromosomes—poleward (P movements) and away from the pole (AP movements)—are coordinated (Skibbens *et al.*, 1993). This coordination maintains elevated tension between sister kinetochores as they oscillate back and forth across the spindle equator (Waters *et al.*, 1996). In $SMC2^{OFF}$ cells, the movements were uncoordinated, and single kinetochores underwent extended P "excursions," moving out from the bulk of the chromosome by up to ~2 μm, trailing a thin chromatin thread (Figure 1, b–e; Supplementary Movies 1 and 2). This extensive centromere stretching was reversible. Thus, as in HeLa cells (Gerlich *et al.*, 2006), condensin is not required for the elasticity of DT40 centromeric chromatin. When nocodazole was added, kinetochore excursions ceased, and the interkinetochore distance of bioriented chromosomes in $SMC2^{OFF}$ cells decreased to the control rest length of $SMC2^{ON}$ cells observed in fixed preparations (Supplementary Movie 3).

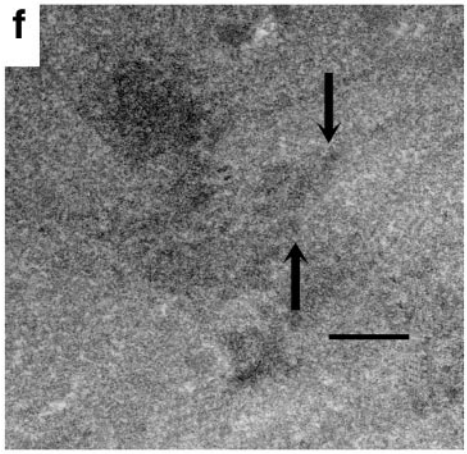
Kinetochore velocities measured during P excursions were 2.1 times faster in $SMC2^{OFF}$ cells than in wild type (3.1 ± 1.2 vs. 1.5 ± 0.8 μm/min). The compensatory antipoleward (AP) returns were also 1.8 times faster (2.8 ± 2.0 vs. 1.6 ± 0.65 μm/min; Figure 1, f and g). These somewhat higher velocities probably reflect a decreased resistance (increased compliance) of the centromeric chromatin to spindle forces. However, a lack of pauses or less frequent switching between directions of motion relative to the rate of image capture when filming in the absence of condensin could also contribute to the apparent increased velocity.

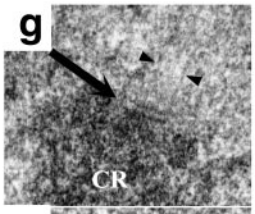
Several lines of evidence indicate that condensin depletion selectively effects the inner centromeric chromatin but not the specialized structures or chromatin of the DT40 kinetochore. 1) CENP-H and CENP-A (markers for the inner kinetochore) localize as discrete spots that are not distorted in kinetochores undergoing excursions (Figure 2, a and b). CENP-A is a modified histone H3 specific for inner kinetochore chromatin (Warburton *et al.*, 1997; Marshall *et al.*, 2008), and CENP-H purifies with CENP-A containing mononucleosomes in vitro (Foltz *et al.*, 2006). 2) Although we cannot exclude that levels of some kinetochore proteins may differ in condensin-depleted kinetochores, loss of condensin had no detectable effect on the absolute number of CENP-H molecules per kinetochore (29 in $SMC2^{ON}$; 31 in $SMC2^{OFF}$) measured by quantitative fluorescence (Figure 2e) relative to the amount of Ndc80 at budding yeast kinetochores (Joglekar *et al.*, 2006). 3) The ratio of CENP-A to CENP-H was unaltered in the presence and absence of condensin (Figure 2e). 4) Localization of Hec1 in the outer kinetochore plate (DeLuca *et al.*, 2005) relative to CENP-A was unchanged by condensin depletion. The average distance between centroids of CENP-A-GFP and Hec1 staining was 60 nm for $SMC2^{ON}$ kinetochores and 66 nm for kinetochores undergoing P excursions in $SMC2^{OFF}$ cells (Figure 2, c and d). 5) EM revealed normal plates, even in kinetochores undergoing P excursions (Figure 2, f–h). 6) DT40 kinetochore plates had an unexpectedly small number of associated kMTs (~4), but this was the same in the presence and absence of condensin

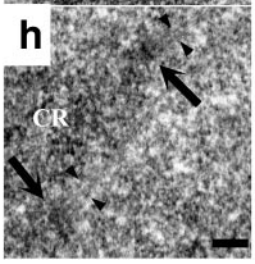


e

	SMC2ON	SMC2OFF
Ratio CENP-H:GFP/ Ndc80	0.222	0.244
CENP-H Molecules/Kt	29	31
Ratio CENP-A:GFP/ CENP-H	0.29 ± 0.06	0.23 ± 0.07







i

	microtubules per kinetochore	range	number of kinetochores	number of cells
SMC2ON	4.3 ± 1.1	3 - 7	32	2
SMC2OFF	4.3 ± 1.2	2 - 7	49	3

Figure 2. Kinetochore structure is preserved in the absence of condensin. (a and b) Relative localization of CENP-A and CENP-H:GFP in SMC2^{ON} (a) and SMC2^{OFF} (b) metaphase cells. Inset in b: kinetochore excursion (arrow) stained for CENP-A (red) and CENP-H:GFP (green). (c and d) Colocalization of Hec1 and CENP-A:GFP in SMC2^{ON} (c) and SMC2^{OFF} cells (d). Inset in d, kinetochore excursion (arrow) stained for Hec1 (red) and CENP-A:GFP (green). Scale bar, 5 μm. (e) Absolute numbers of CENP-H and relative numbers of CENP-A are conserved in SMC2^{ON} and SMC2^{OFF} kinetochores.(f) Low-magnification EM image of an SMC2^{OFF} metaphase cell. Upper arrow, kinetochore excursion; lower arrow, kinetochore at the metaphase plate. (g) Kinetochore from a SMC2^{ON} cell: inner and outer plates (arrow) and associated MTs (arrowheads). (h) Higher magnification of the two SMC2^{OFF} kinetochores indicated in panel f. The outer plate (arrow) and attached MTs (arrowheads) of both kinetochores are indicated. Scale bars, (f) 1 μm; (h) 200 nm. (i) The number of MTs per kinetochore is the same in SMC2^{ON} and SMC2^{OFF} cells. This number was determined from chromosomes aligned at metaphase in serial EM sections of SMC2^{ON} and SMC2^{OFF} cells.

(Figure 2i). Indeed, with a number of MTs per kinetochore similar to that in *S. pombe* (Ding *et al.*, 1993), DT40 cells may provide a system that is naturally more sensitive to factors that influence kMT interactions than other vertebrate kinetochores, which have roughly fivefold more MTs per kMT fiber. In summary, neither the inner nor the outer kinetochore showed detectable structural alterations in DT40 cells depleted of condensin.

These results suggested that vertebrate centromeres have a robust kinetochore platform consisting of a CENP-A chromatin core beneath that is elastic inner centromeric chromatin that links sister kinetochores together. Such a two-domain model was further supported by in vitro experiments in which wild-type chromosomes were placed in a buffer (10 mM triethanolamine:HCl, pH 8.5, 10 mM NaCl, and 1 mM EDTA) designed to be inefficient at neutralizing the excess negative charges on the DNA that remain in chromatin. In this buffer, chromatin higher-order structure unfolds to beads-on-a-string nucleosomes (Earnshaw and Laemmli, 1983), and mitotic chromosome structure was completely disrupted (Figure 3a; Earnshaw and Laemmli, 1983; Hudson *et al.*, 2003). Under these conditions, CENP-A–containing chromatin retained its condensed morphology, whereas the inner centromeric heterochromatin progressively unfolded (Figure 3b). With longer incubations, the CENP-A–containing chromatin also unraveled into strings of dots, consistent with it being a specialized chromatin domain.

These results suggest that the centromere rest length is likely set by chromatin higher-order structure in vertebrates as it is in budding yeast (Bouck and Bloom, 2007) and is independent of condensin. Together, the data thus far suggest that depletion of condensin affects the mechanical properties of the inner centromeric chromatin rather than the structure of the kinetochore itself.

ATPase Activity of Condensin Is Essential for the Maintenance of the Rigidity of the Centromeric Chromatin

Efforts to determine the role of condensin at centromeres by following individual kinetochore movements were complicated by the fact that DT40 cells contain more than 150 kinetochores. To address the molecular mechanism for condensin stabilization of centromeric chromatin under tension, we generated a simplified in vivo model system for moni-

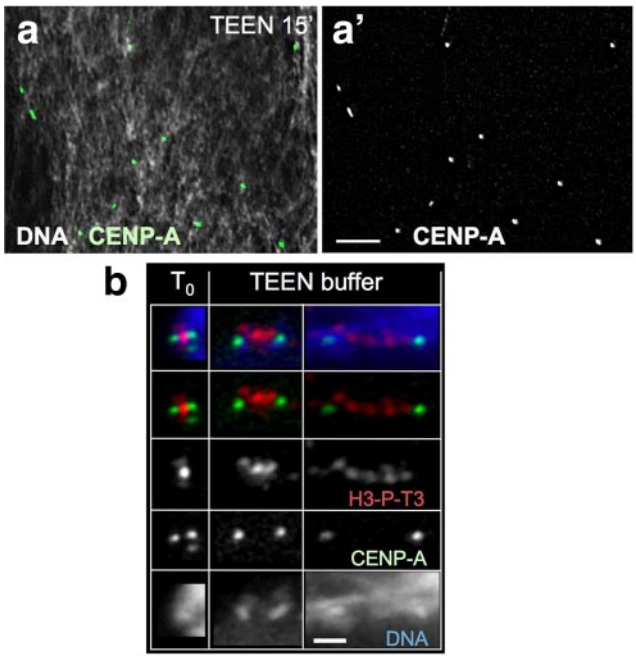


Figure 3. CENP-A:GFP containing chromatin is more resistant to unfolding than centromeric heterochromatin. (a–a′) After 15 min in TEEN buffer the majority of CENP-A signals are still compact (dot-like). Scale bar, 5 μm. (b) Kinetochore domains of mitotic chromosomes containing CENP-A:GFP (green) are more resistant to unfolding in low ionic strength TEEN buffer than bulk chromatin or inner centromere chromatin stained for H3-P-T3 (red). Scale bar, 2 μm.

toring sister chromatid movements in metaphase. We isolated a SMC2$^{ON/OFF}$ cell line with a lacO array inserted in the pericentromeric region of a microchromosome (Figure 4a, CEN). This pericentric LacO array underwent movements resembling those of kinetochore pairs in living SMC2^{ON} and SMC2^{OFF} cells (Figure 4c; Supplementary Figure S2, a and c; Supplementary Movies 4 and 5), and on average, the LacI:GFP signals on sister chromatids were further apart in SMC2^{OFF}:CEN cells fixed at metaphase (Figure 4b). Similar separation of pericentric regions was previously observed in wild-type budding yeast for reporter arrays adjacent to centromeres (Goshima and Yanagida, 2000; He *et al.*, 2000; Pearson *et al.*, 2001), but has not previously been observed in vertebrates.

Kymographs revealed the dynamic separation between the LacI:GFP^{CEN} signals (Figure 4c), and their uncoordinated behavior was readily observed in movies of SMC2^{OFF}:CEN cells (Supplementary Movies 4 and 5). Remarkably, the loss of condensin caused an approximately twofold increase in the maximum separation of the chromosomal region linking the two LacO arrays (Figure 4d). Thus, in this simplified system pericentric chromatin accurately reproduces the behavior of kinetochores. As with kinetochores, the excursions were reversible and MT-dependent. They were abolished by nocodazole (Figure 4c; Supplementary Figure 2f, and Supplementary Movie 6) or the RNAi knockdown of Hec1 (Figure 2b; Supplementary Figure 3g and Supplementary Movies 7–9). The oscillatory movements were unaffected by the addition of proteasome inhibitor MG132 (Figure 4c; Supplementary Figure 3e and Supplementary Movie 10). In controls, a LacO array integrated on the arm of a macro-chromosome (Figure 4a, ARM) maintained a constant relative distance between sister chromatids during metaphase in cells lacking condensin (Figure 4c; Supplementary Figure 3, b and d, and Supplementary Movies 11 and 12). This distance was larger than that in cells expressing SMC2 because, unlike inner centromeric chromatin, arm chromatin is 40% less compact in SMC2^{OFF} cells (Hudson *et al.*, 2003; Vagnarelli *et al.*, 2006).

To begin to address the underlying mechanism by which condensin regulates centromere stiffness, we asked whether SMC2 ATPase activity is required for normal behavior of kinetochores under tension at metaphase. One strength of the DT40 cell conditional knockout system is that it is possible to perform complementation studies under conditions where the wild-type protein is essentially undetectable. Indeed, expression of wild-type SMC2 driven by a fragment of the endogenous SMC2 promoter restored the shorter distance between the two centromere-proximal sister loci in SMC2^{OFF}:CEN cells (Figure 4e). However, SMC2^{S1086R}, a mutant capable of assembling a condensin complex that targets to chromosomes (Supplementary Figure S1) but that lacks ATPase activity (Hudson *et al.*, 2008) failed to rescue the spacing between sister kinetochores (Figure 4e). Therefore, normal stiffness of the centromeric chromatin requires SMC2 ATPase activity.

Loss of topoisomerase II (topo II) can reduce the interkinetochore distance in metaphase (Spence *et al.*, 2007), and topo II can influence the longitudinal elasticity of mitotic chromosome arms (Kawamura and Marko, personal communication; Marko, 2008). Indeed, treatment with topo II inhibitor ICRF 159 slightly reduced the spacing between sister kinetochores, even in the presence of condensin (Figure 4e). However, addition of the drug failed to restore normal centromere stiffness to SMC2^{OFF} cells lacking condensin. Thus, catenation regulated by topo II can have a minor effect on the compaction of inner centromeric chromatin, but is not a major factor regulating its compliance.

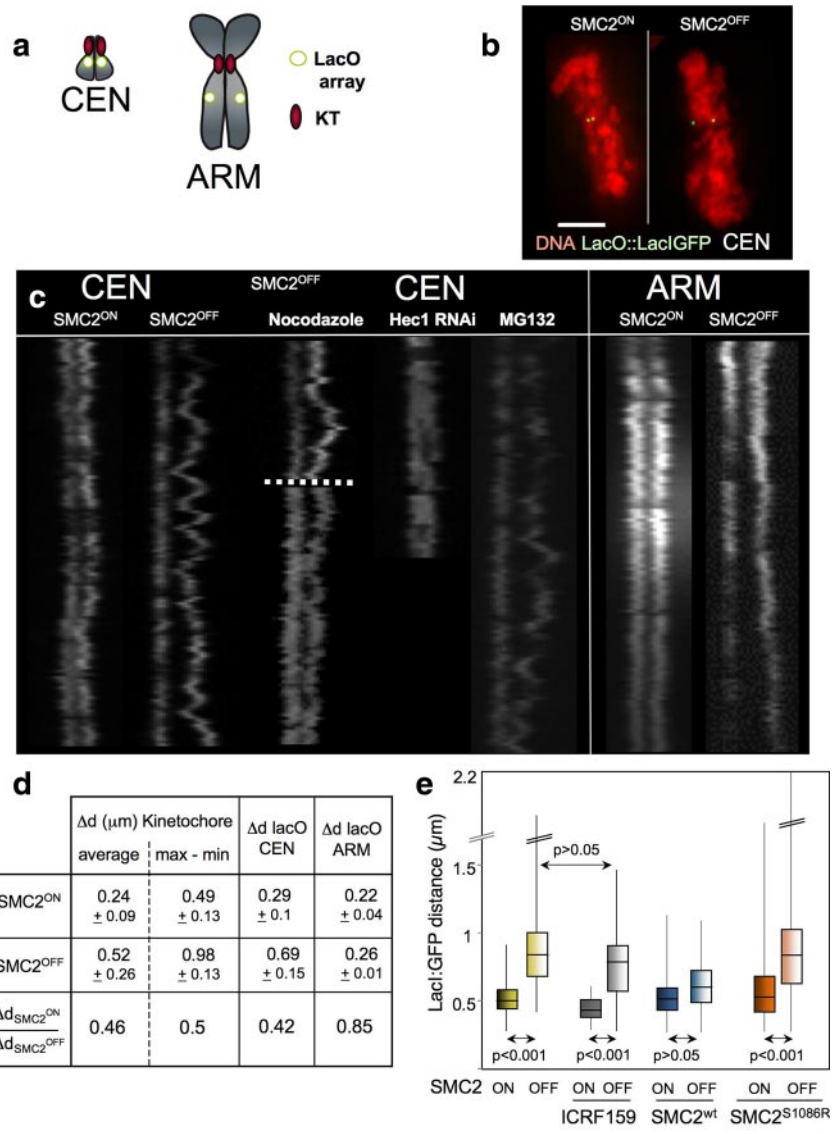
Proper Centromeric Stiffness Is Required for a Timely Silencing of the Spindle Assembly Checkpoint

A clue to the functional significance of the regulation of centromere stiffness by condensin was provided by the observation that in unperturbed cell cycles, the mitotic index in SMC2^{OFF} cells is higher (7.7 $\pm$ 3.5%) than in SMC2^{ON} cells (3.1 $\pm$ 1.2%), consistent with a mitotic delay (Figure 5, a and b, 0 time point). These cells accumulate in late prometaphase/metaphase before entering anaphase (Figure 5h). Time-lapse imaging cells revealed that condensin-depleted cells do not have problems in chromosome congression or in the maintenance of chromosome alignment (Figure 5k). Nonetheless, the mitotic delay appears to be due to activation of the spindle checkpoint.

SMC2^{OFF} cells activate the spindle checkpoint normally in the presence of colcemid or taxol (Figure 5, a and b). The mitotic delay observed in otherwise unperturbed cell cycles appears to arise from sustained checkpoint activation, as we observed an increase in the number of SMC2^{OFF} cells with aligned metaphases having two or more Mad2:GFP-positive kinetochores (Figure 5, c–g). However, kinetochores undergoing P excursions lacked detectable Mad2 or BubR1, or the MT de-polymerase MCAK (Figure 5, f and j), suggesting that KTs engaged in these extended movements exhibit a full MT occupancy. Instead, the Mad2-positive kinetochores were present on chromosomes apparently aligned on the metaphase plate.

Condensin Sets the Stiffness of the Centromeric Chromatin

If we model the inner centromeric chromatin as a spring using Hooke's law as a simplifying assumption (Gardner *et*



	Δd (μm) Kinetochore		Δd lacO CEN	Δd lacO ARM
	average	max - min		
$SMC2^{ON}$	0.24 ± 0.09	0.49 ± 0.13	0.29 ± 0.1	0.22 ± 0.04
$SMC2^{OFF}$	0.52 ± 0.26	0.98 ± 0.13	0.69 ± 0.15	0.26 ± 0.01
$\frac{\Delta d_{SMC2^{ON}}}{\Delta d_{SMC2^{OFF}}}$	0.46	0.5	0.42	0.85

Figure 4. Pericentromeric movements require dynamic MTs and SMC2 ATPase activity. (a) Diagram showing LacO array integration sites in two different $SMC2^{ON/OFF}$ cell lines: CEN (integration in pericentromeric chromatin) and ARM (integration in the q arm of macro-chromosome). (b) Aligned metaphases with LacI-GFP signals in the same focal plane for $SMC2^{ON}$ and $SMC2^{OFF}$ cells. Scale bar, 5 μm. (c) Kymographs of the LacO array movements. In $SMC2^{OFF}$ cells pericentromeric chromatin undergoes excursions similar to those of sister kinetochores. These movements are abolished by nocodazole (dotted line shows time of addition) and Hec1 RNAi; they are unaffected by the proteasome inhibitor MG132. The ARM locus exhibits an increased spacing but no oscillations in $SMC2^{OFF}$ cells. (d) Summary of the average Δd and Δd_{max} values and their ratios for all loci determined from live cell imaging data. The Δd_{max} values for the kinetochores and pericentromeric integration are increased by 50 and 58%, respectively, in the absence of condensin, whereas no significant changes are obtained for the ARM integration. (e) Distribution of distances between the LacI:GFP signals in fixed metaphases of $SMC2^{ON}/SMC2^{OFF}$:CEN cells incubated with 20 μM MG132. Topo II inhibition (ICRF 159) does not restore the normal interlocus spacing. $SMC2^{wt}$ restores the normal interlocus spacing distribution but an ATPase-deficient SMC2 point mutant ($SMC2^{S1086R}$) does not; n = 90. $p < 0.001$; Mann-Whitney U test.

al., 2005), then $F = -k\Delta d$, where F is the force applied, k is the spring constant or stiffness of the spring, and Δd is the distance stretched beyond the rest length. We postulate that F_{MAX} is likely to be approximately equivalent in the presence and absence of condensin because the number of kMTs was the same in $SMC2^{ON}$ and $SMC2^{OFF}$ cells (Figure 2i) and because kinetochores, spindles, and the rest length of the centromeric chromatin between sister kinetochores were close to normal in $SMC2^{OFF}$ cells (Figure 2; Supplementary Figure 1, d and e). Recent studies in budding yeast found that the nucleosome architecture of chromatin regulates the rest length but not the stiffness of centromeres (Bouck and Bloom, 2007).

The average extent of P excursions (Δd) in $SMC2^{OFF}$ cells (0.52 ± 0.26 μm) was about two times that observed in $SMC2^{ON}$ cells (0.24 ± 0.09 μm; Figures 4d and 6a; Supplementary Figure S2a). Therefore, according to Hooke's law the spring constant of centromeres lacking condensin is 0.46 that of wild-type centromeres. Strikingly, a nearly identical value was obtained when we analyzed the movements of the centromere-linked LacO-CEN locus (Figure 4d). This is the first evidence for a protein complex affecting specifically the spring constant (stiffness) of centromere chromatin.

DISCUSSION

Using a conditional knockout for the SMC2 subunit of the condensin complex coupled with live cell imaging, we have shown that the ATPase activity of SMC2 is required for the normal stiffness of inner centromeric chromatin when bioriented kinetochores are subjected to MT pulling forces in metaphase. We also found that kinetochores behave in DT40 cells as structurally independent domains that are linked by the elastic chromatin at the inner centromere. These dynamic properties of the centromeric chromatin are linked to spindle checkpoint activation and silencing. Using this system, we have shown that a weak centromeric spring causes delay in mitotic progression due to prolonged checkpoint activation.

The results obtained in our experimental model for observing kinetochore dynamics are in agreement with a previous study in which condensin depletion caused a

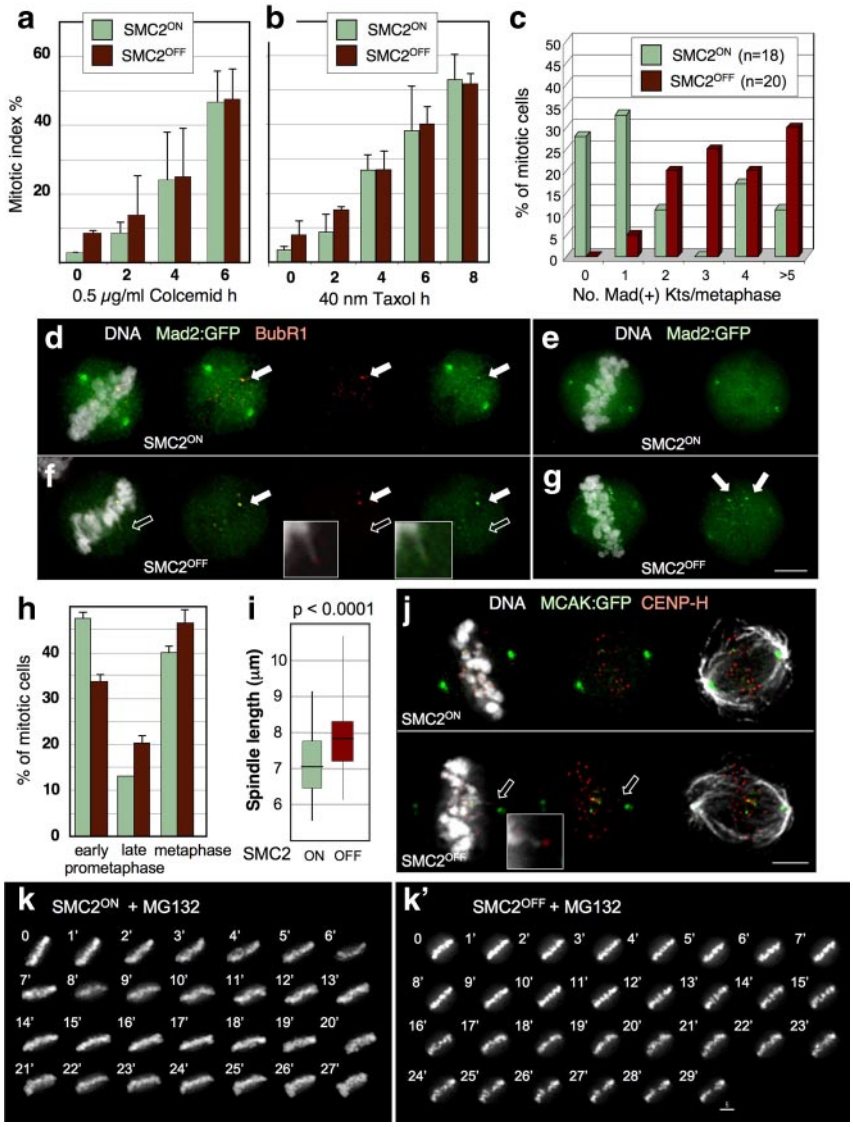


Figure 5. SMC2^{OFF} cells exhibit persistent spindle checkpoint activation. (a and b) SMC2^{OFF} cells can activate the spindle checkpoint normally in presence of colcemid (a) or taxol (b). n = 500 in each of three experiments. (c) Quantification of Mad2-positive kinetochores per metaphase. (d and f) Kinetochores of SMC2$^{ON/OFF}$ cells undergoing poleward excursions are negative for Mad2 and BubR1 (empty arrow and inset). Mad2:GFP-positive kinetochores are BubR1-positive in SMC2$^{ON/OFF}$ cells (filled arrows). (e and g) Staining SMC2$^{ON/OFF}$ kinetochores in aligned metaphases for Mad2. Scale bar, 5 μm. (h) Cells without condensin show an increase in late prometaphase and metaphase cells. n = 50 in each of three experiments. (i) SMC2^{OFF} spindles are longer than SMC2^{ON} spindles; n = 110. (j) Kinetochores of SMC2^{OFF} cells engaged in poleward excursions are MCAK-negative (inset and empty arrow). Scale bar, 5 μm. (k and k') Chromosomes of SMC2^{ON} and SMC2^{OFF} cells maintain a stable metaphase alignment in the presence of MG132.

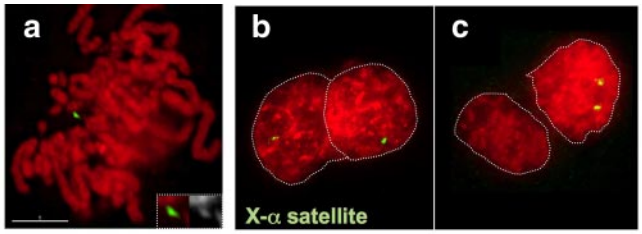
reversible deformation of the centromeric chromatin accompanying uncoordinated sister kinetochore movements (Gerlich *et al.*, 2006). We could not confirm results reported for *Drosophila*, in which similar kinetochore movements were observed, but where the distortions of the centromeric chromatin were irreversible (Oliveira *et al.*, 2005).

Kinetochores Appear Structurally and Functionally Normal in Condensin-depleted DT40 Cells

Several studies have reported altered centromere/kinetochore structure in the absence of condensin. In yeast, condensin depletion causes loss of Cse4 from centromeres (Yong-Gonzalez *et al.*, 2007). In human cells, RNAi for condensin caused aberrations in CENP-E and CREST signal geometry in one study (Ono *et al.*, 2004) but no evident distortion of CENP-A signals in another (Gerlich *et al.*, 2006). In *Xenopus* egg extracts, immunodepletion of condensin caused abnormal CENP-E localization (Wignall *et al.*, 2003), and in *Drosophila* condensin RNAi, CID (the CENP-A homologue) was found to be distorted in metaphase (Jäger *et al.*, 2005). Distortion of the kinetochore was also observed in the holocentric chromosomes of *Caenorhabditis elegans* after condensin RNAi (Hagstrom *et al.*, 2002).

We show here that in DT40 cells, kinetochore overall structure is maintained after the depletion of condensin, as indicated by serial-sectioning EM and analysis of the distribution and/or copy number of several key kinetochore components. Likewise, kinetochore function is maintained, as indicated by spindle checkpoint activation and silencing when the kinetochores are engaged in poleward excursions at high degrees of centromere stretch. Furthermore, we showed previously that kinetochores in DT40 cells lacking detectable condensin segregate to opposite spindle poles during anaphase even when the chromatin trailing behind them becomes grossly distorted (Hudson *et al.*, 2003; Vagnarelli *et al.*, 2006). This is reminiscent of results in *C. elegans* showing that even though kinetochores in embryos lacking SMC2 were abnormal in metaphase, they adopted a normal morphology by anaphase (Kaitna *et al.*, 2002). Furthermore, if the targeting subunit RepoMan is prevented from recruiting protein phosphatase 1 to the chromatids during anaphase, then the entire process of anaphase chromatid segregation appears to be completely normal in DT40 cells lacking detectible condensin (Vagnarelli *et al.*, 2006).

The differences between the various studies are likely to have at least two explanations. First, DT40 kinetochores bind



d

	MC SEGREGATION %				N
	1:0	1:1	2:0	other	cells
SMC2^{ON}	3.3	94	1.1	1.6	458
SMC2^{OFF}	2.7	95	1.5	0.8	524

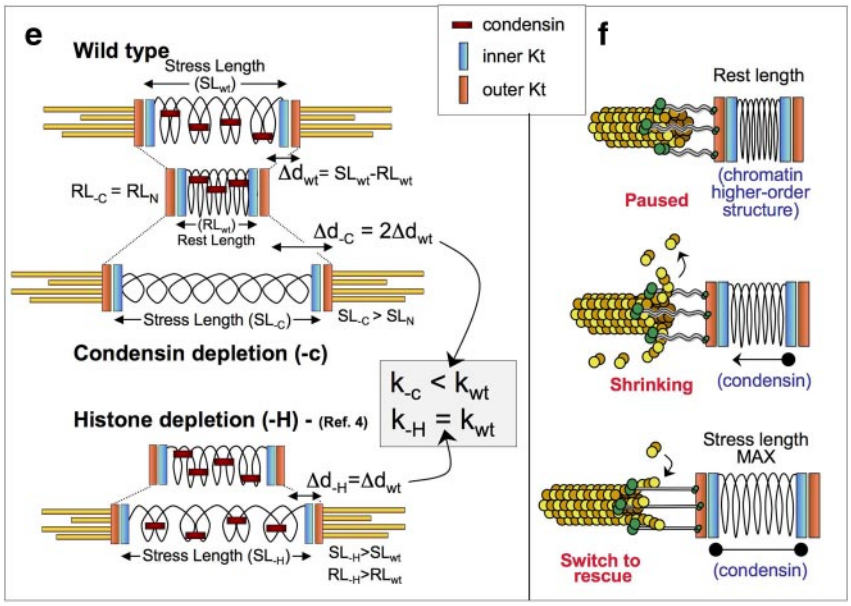


Figure 6. Proposed role of condensin in regulating the compliance and MT dynamics at centromeres. (a–c) SMC2$^{ON/OFF}$ cell line carrying a human minichromosome was used to test the frequency of mis-segregation in the presence and absence of condensin. (a) Chromosome spread hybridized with a chromosome X-specific α-satellite probe; (b and c) binucleate cells showing two different types of segregation 1:1 (b) and 2:0 (c); (d) quantification of the experiment. (e) Without condensin, unstressed centromeric chromatin is as compact as wild type (rest length). On MT attachment, centromeres in SMC2^{OFF} cells deform twice as much as SMC2^{ON} centromeres (Δd). H3 or H4 depletion increased the rest length but the deformation due to spindle forces was unaltered (Bouck and Bloom, 2007). (f) Hypothetical model linking centromere chromatin elasticity and kinetochore MT dynamics.

for four- to fivefold fewer MTs than the other metazoan kinetochores examined, and this could render them less susceptible to distortion in the absence of condensin. Second, the present study and that of Gerlich *et al.* (2006), which also failed to describe kinetochore structure abnormalities after condensin RNAi in human cells, were conducted mostly on live cells. Our analyses on fixed samples were performed using optimized fixation conditions that preserved the structure of both the chromatin and the kinetochore. This was particularly significant in this case because we have shown previously that condensin-depleted chromosomes are exquisitely sensitive to fixation conditions (Hudson *et al.*, 2003), and disruption of kinetochore structure may have occurred in other studies during sample preparation.

An altered structure of the centromere might be expected to produce an increase in merotelic attachments and consequent chromosome mis-segregation. We did not observe this when monitoring the segregation of either lacI-GFP-tagged loci (Hudson *et al.*, 2003; Vagnarelli *et al.*, 2006) or a human mini-chromosome in cells with or without condensin (Figure 6, a–d), nor did we see any evidence of lagging kinetochores at anaphase in our earlier studies of the condensin knockout cells (Hudson *et al.*, 2003; Vagnarelli *et al.*, 2006). Because kMT attachment is believed to be a stochastic process, the probability of merotelic attachment may be less for kinetochores with fewer MT-binding sites. Regardless, our data show conclusively that condensin is not an obligate component of a system preventing merotelic attachments in vertebrate kinetochores.

Mitotic Delay in Condensin-depleted Cells Is Caused by Prolonged Activation of the Spindle Assembly Checkpoint

Our analysis suggests that centromere stretch has a biological function in regulating MT attachment to kinetochores. While this manuscript was under revision, two articles were published showing that intrakinetochore stretch during mitosis is important for silencing the spindle checkpoint (Maresca and Salmon, 2009; Uchida *et al.*, 2009). These results could give the impression that centromere stretch between sister kinetochores is not required to silence the checkpoint. However, such an interpretation would be misleading. Indeed, many studies over the years have shown that tension can stabilize MT attachment to kinetochores and promote MT growth (Nicklas and Koch, 1969; Rieder and Salmon, 1994; Inoue and Salmon, 1995; Skibbens *et al.*, 1995; Nicklas *et al.*, 2001; Gardner *et al.*, 2005; Figure 6, e and f).

Thus, although tension between sister kinetochores may not directly silence the spindle checkpoint signaling cascade (Maresca and Salmon, 2009; Uchida *et al.*, 2009), this tension is likely to lessen the probability that kinetochores will release their MTs (Nicklas *et al.*, 2001). Of course, kinetochores

that release their MTs because of a lack of tension do activate the spindle checkpoint. Therefore, there is likely to be a critical, if indirect, link between the degree of interkinetochore centromere tension and activation/inactivation of the spindle checkpoint.

We did not analyze intrakinetochore stretch in the present study; however, our data are consistent with two possible models to explain the checkpoint activation and mitotic delay consequent upon condensin depletion. First, condensin depletion may affect tension within the kinetochore itself, thereby promoting checkpoint activation as suggested recently (Maresca and Salmon, 2009; Uchida *et al.*, 2009). Second, the alteration in compliance of the centromeric chromatin that occurs upon condensin loss may create a gradient of tension within the spindle. This could cause kinetochores to release their MTs and activate the spindle checkpoint when tension is below a threshold, particularly in a sensitized system such as DT40 cells with only four MTs per kinetochore. Indeed, we observed an increase in Mad2-positive kinetochores located near the metaphase plate in the region where we would expect spindle tension to be lowest. Mad2 localization to kinetochores is widely accepted to be diagnostic of a lack of MT occupancy, and our result suggests that the kinetochores of chromosomes near the metaphase plate are more likely to release their MTs after condensin depletion.

In condensin-depleted cells the kinetochores that are engaging in the most obviously abnormal behavior—the poleward excursions—are not those that are signaling to the spindle checkpoint. We explain this by suggesting that even weakened springs can produce the same level of tension as stronger springs—this merely happens at a greater Δd (stretch) and is consistent with the observation that spindles are slightly longer in $SMC2^{OFF}$ cells (Figure 5i). Thus, the reduced stiffness of centromeric chromatin in condensin-depleted cells could create a gradient of tension: lowest near the surface of the centromere and progressively higher as the kinetochore is stretched further poleward. Because Aurora B activity is high on the chromosomes (Fuller *et al.*, 2008), regions of lowered tension might be expected to favor kMT detachment and spindle checkpoint activation (King and Nicklas, 2000). Indeed, kinetochores undergoing excursions might be predicted to have particularly stable MT attachments, because they are located furthest from the Aurora B in the inner centromere (Cimini *et al.*, 2006).

Decreased tension could also occur when uncoordinated sister kinetochores engage in simultaneous AP movements for longer than normal periods; however, this would not a priori show any preference for position on the spindle. Reproducibly, the Mad2-positive kinetochores were located close to the metaphase plate where tension would be expected to be lowest according to the gradient model.

SMC2 ATPase Activity Determines the Compliance of the Centromeric Chromatin

The loss of condensin causes an approximately twofold decrease in the stiffness of the centromeric "spring" when measured by two quite distinct reporter loci (Figure 4g). The underlying molecular mechanism is unknown, but SMC2 depletion causes changes in the localization and chromosomal association of DNA topo II alpha, KIF4A and a number of other chromosome scaffold components (Hudson *et al.*, 2003; Gassmann *et al.*, 2004). Thus, it is possible that condensin depletion alters the distribution or function of proteins involved in establishing or regulating sister chromatid cohesion, such as cohesin or Sgo1.

The twofold decrease in spring constant is consistent with a halving of the structural links between kinetochores. This suggests an obvious parallel with the ability of SMC-based cohesin rings to link pairs of chromatin fibers together (Nasmyth and Haering, 2005). Full understanding of the organization and dynamics of the centromere "spring" will therefore require a detailed elucidation of the mechanism of centromeric chromatin binding by condensin.

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